STUDIES OF SMALL INTESTINAL MUCOSAL KINETICS

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1970

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A. Conventionalization of the Germfree Mouse

B. Effects of an Oral Antibiotic Regimen:
    Morphological and Functional Correlations

By

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B.S., University of Notre Dame, 1966

A thesis presented to the faculty of the Yale University
School of Medicine in partial fulfillment of the require-
ment for the degree of Doctor of Medicine.

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1970

We must clear our minds from all causes that blind people to the truth.

must clear our minds from all cause that blind people to the truth,

To Lucy, in all a companion and in all an inspiration.

To Lucy, in all...

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Cellular kinetics and the mechanisms which control cell proliferation attract a great deal of medical interest, imagination and research, ranging from fertilization and embryological development to aging and malignancy. Many questions have been asked and answers gained but many more need follow as fundamental insight into cell reproduction and control awaits discovery.

A recently developed and highly useful tool in the study of cellular kinetics, radioautography, follows morphology dynamically and thus introduces a temporal dimension into histological study (85). A film-stripping technique was first developed (87), subsequently improved by the more rapid and convenient dipping technique (75). Isotopes of varying intensities and purposes were utilized but tritiated thymidine was found to be the most suitable for labeling DNA in the cells' nuclei (23).

The kinetics of various systems has been studied by radioautography. The mucosa of the gastrointestinal tract has proved one of the most instructive because of its ordered histology, its rapid turnover, and its sensitivity (7,10,18,27,96,98,99,100,105). But the gastrointestinal mucosa has a unique environment in the foreign world of its lumen, inhabited by voluminous secretions, engulfed travelers as varied and bizarre as the psychic mechanisms and habits selecting them and, of course, astronomical numbers of micro-organisms. What effect has this environment, especially in terms of its bacterial colonization, upon mucosal kinetics?

In this spirit gut kinetics was investigated by radioautography with tritiated thymidine. But to investigate dynamic morphology alone would be to substitute one dimension for another, that is, time for function. Anatomy not only is, it functions. Thus some aspects of gut physiology were also investigated.

The gut absorbs. Studies of intestinal transport have been simplified by a technique employing everted sacs of small intestine and measuring the amount of the labeled substance which is actively absorbed (11,155). An essential amino acid, 1-methionine, was the nutrient tested in this study.

Enzymes are essential in biological reactions. Histochemical staining methods, employing frozen tissue sections (19,20,69,115,135), were utilized to study intestinal mucosal enzymes. Also the disaccharidases of the brush border were assayed by spectrophotometric means (29). Thus, gut dynamic morphology with physiological and enzymatic correlates was investigated in relation to alterations in the intestinal bacterial flora.

Colonization was approached by two means. First, germfree animals, now widely available (117) and easily usable (5,152) and known to have a unique gut morphology and physiology (3,56,65,103) were employed. Second, animals were treated with a wide-spectrum antibiotic regimen (49,55) to modify and limit the micro-organisms of the gut. Recent advances in qualitative and quantitative fecal culturing techniques (13,51) made it possible to follow changes in bacterial flora.

Two related experiments were performed with mice. In the first study, small intestinal mucosal kinetics and fecal flora were followed from the germfree state through the conventionalization process in order to ascertain the time needed for these parameters to reach the conventional state. In the second study, the effects of an antibiotic regimen of neomycin and penicillin were examined in order to ascertain the consequences of intestinal microfloral reduction. Small intestinal mucosal cell kinetics, fecal flora, 1-methionine transport, lactase and sucrase activity, and histochemical

levels of acid and alkaline phosphatase, non-specific esterase, and reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), lactic and succinic dehydrogenase were followed during the antibiotic treatment.

SMALL INTESTINAL MUCOSAL CELL PROLIFERATION AND BACTERIAL FLORA
IN THE CONVENTIONALIZATION OF THE GERMFREE MOUSE

Intestinal mucosal cells, originating in the crypts, migrate up the villi of the small intestine and slough off into the lumen (88,26). Studies of the kinetics of these events have revealed a differential rate of cellular renewal between the intestinal mucosal cells of germfree animals and those of conventional animals (2,91). The time for migration of the mucosal cells from the crypt to villus tip is twice as long in the germfree animal (2,91). The absence of intestinal bacterial flora in the germfree animal may account for this difference in dynamic morphology.

Recent advances in intestinal and fecal culturing techniques have provided information about the intestinal bacterial flora under normal and experimentally altered conditions (22,39). Changes in micro-organisms have been followed after contamination in germfree (137) and newborn animals (136,41,110,145).

The present study was designed to follow the changes in cellular renewal in going from a germfree system to a conventional one as a function of time with reference to the intestinal microbial colonization. Experiments involving both germfree and conventional animals have utilized conventionalized (formerly germfree) animals as the control subjects. But the possibility of altering cellular kinetics of the intestinal epithelium and the time span necessary for the alteration have not previously been investigated.

Materials and Methods

Animals:

Thirty-five male and thirty-five female CFW mice (Carworth Farms, New City, New York) born and reared under standard germfree conditions were randomly divided into two groups each containing equal numbers of males and females. One group of 16 animals was maintained in plastic cages in a

Snyder (Trexler type, Snyder Mfg. Co., New Philadelphia, Ohio) flexible-film isolator under standard germfree conditions (151). They were given steril-ized drinking water and fed a sterilized commercial diet (Purina Laboratory Chow, Special Formula 5010C, Purina Laboratories, St. Louis, Missouri) ad libitum. In order to ascertain that a germfree environment was being main-tained, bacterial cultures of the isolators, the bedding, and the animals' fecal dropings were taken both on arrival of the animals and upon each entrance into the isolators.

Another group of 54 mice was removed from the germfree isolators and placed in a conventional animal room. These animals received the same sterilized diet and drinking water ad libitum as the germfree animals. Fresh stools of selected conventionalized mice were obtained daily for culture of both anaerobic and aerobic micro-organisms on selective media.

Radioautography:

Thirty microcuries (6.7 curies/millimole in 0.3 mg sterile water) of sterile thymidine-methyl-H^3 (New England Nuclear Corp., Boston, Mass.) was injected intraperitoneally. The injection of the germfree mice was performed in the isolator by admitting the sterile thymidine vials through a transfer lock sterilized with 2% peracetic acid. Animals were removed from the iso-lators only immediately before sacrifice.

The schedule of the labeled thymidine injections is enumerated in Table I. On day 0, for instance, all the germfree mice were injected as were numbers 1 to 6 of the animals taken from the germfree environment. On day 1, 24 hours after conventionalization, mice numbers 7 to 12 were similarly injected. On day 2, and subsequently, other animals in the con-ventionalized group were injected, until the eighth day. On the same table

is listed the time after injection when the appropriate mice were sacrificed and specimens removed for study. Thus, for each day after conventionalization, radioautography was done 24, 48, and 72 hours after the administration of the labeled thymidine. In this manner, cell population kinetics in the mouse ileum were studied from one to ten days after conventionalization from a germfree environment.

Each mouse was sacrificed with ether anesthesia and a five cm segment of the small bowel was removed 5 cm from the ileocecal junction. The intestinal specimens were first rinsed with Krebs buffer (pH=7.4) and then fixed in a solution of 10% neutral formalin. Each specimen was divided into three equal segments, embedded in paraplast, and cross-sectioned at 4 microns at three comparable levels separated by at least 100 microns. The slides were then coated with Kodak NTB-2 nuclear track emulsion by the dipping method (76,82) at 80% humidity and 28°C. The slides were stored with the tissue section up in black boxes and maintained in a dark room at 4°C. After 33 days of exposure, the slides were developed and then stained with hematoxylin-light green. After the slides were coded, twenty to thirty "ideally" (mid-longitudinally) sectioned villi were selected for quantitation from each animal. At 400 x magnification the total number of cells lining one side of the section of each villus was counted from crypt to tip. The number of cells behind the leading edge of the labeled epithelium was also counted. Thus, an average villus height and an average labeled height, in terms of epithelial cells in a single-file column, were determined for each animal. The percent value of total cells of the villi labeled was then calculated.

Bacteriology:

Alterations in the bacterial flora in the conventionalized mice were followed by qualitative and quantitative cultures of fecal samples on

selective culture media. 0.1 gm of the stool sample was dispersed and diluted in 9.9 ml of norite A charcoal water and serial dilutions were made. A calibrated platinum loop (delivering 0.01 ml) was used to streak a series of eight culture media from the tube dilutions so that a final quantitative count of total aerobes, total anaerobes, coliforms, Strepto-cocci spp., Bacteroides spp., Clostridia spp., and total microaerophilic and anaerobic lactobacilli was obtained. The details of these methods are described elsewhere (51).

Results

The labeled cells per villus, 24 hours after administration of H^3-thymidine, was nine per cent in the germfree animal as contrasted with 31 per cent in the conventional mouse (Figure 1). Forty-eight, 72, and 96 hours after administration of the H^3-thymidine to the germfree animals, 40, 70, and 92 per cent of the cells, respectively, were labeled (Figure 2). In the conventional counterpart, however, 97 per cent of the cells were labeled by 48 hours (Figure 3). Figure 1 shows the results of the radio-autography studies in the conventionalized animals, up to the eighth day after removal from the germfree environment. The graph reveals a progres-sive increase in the per cent of cells labeled, beginning on the first day of conventionalization. By the fourth day, the per cent of cells labeled 24 hours after administration of the H^3-thymidine was similar to a conven-tional animal. However, as far as the per cent of cells labeled 48 hours after giving the label was concerned, the value reached 90 per cent labeling of the value in the conventional animal on the eighth day of conventionali-zation (Figure 4).

Bacteriologic studies of the germfree housing and fecal matter revealed no bacterial growth in the culture media employed. On fecal cultures of the conventionalized animals both anaerobes and aerobes were recovered one day after conventionalization. By the seventh day, the pattern of bacteria recovered in the stool was akin that of our conventional mice, housed in the same animal room (Table 2).

Discussion

When animals reared under standard germfree conditions were placed in a conventional environment, proliferation of intestinal bacteria occurred within a day. A stable bacterial pattern was cultured in the stool by the third day of conventionalization and by the seventh day it remained qualitatively and quantitatively similar to that of true conventional mice. Temporally related to the changes in intestinal bacteria was the observation that the epithelial cell migration rate doubled over the eight day period from the beginning of conventionalization. Thus conventionalized animals used in germfree research must be considered to be in a state of transition for the first eight days, as far as dynamic small intestinal morphology is concerned. At this time epithelial cell migration and intestinal microflora become similar to those of animals reared under standard conditions. Since the maturity of epithelial cells and their enzyme systems relates to the rate of migration in the villi (113), these changes likely have significance in terms of intestinal digestive and absorptive mechanisms.

Various factors (92,1,140,101,141,70) and substances (Part II of thesis, 86,25,61) affect the mitotic and the migration rates of intestinal epithelial

cells. In older animals, production of cells in the crypts is decreased
and the generation time is increased, while the migration rate on the villus
is decreased (92). In the short period of time involved in this study,
age probably did not play a role in affecting cell kinetics.

An experimentally induced enteric infection with Salmonella typhimurium
caused in the ileal epithelium an increase in the cell renewal rate and a
decrease in the transit time of the epithelial cells from the crypt to
the tip of the villus (1). Similar findings have also been reported in
association with non-enteric infections, as after pneumonococcal septicemia
(140).

Germfree animals have a decreased intestinal epithelial cell migration
rate, as compared to their conventional counterparts. Theoretically, elimina-
tion of intestinal bacteria with antibiotics could change small intestinal
cell kinetics in the conventional animal toward that in the germfree; how-
ever, when neomycin is one of the antibiotics employed, direct effects on
the small bowel occur (Part III of thesis). Epithelial cell migration rate
is decreased after administration of neomycin (Part II of thesis).

A number of studies have been concerned with the changes which may be
induced by the microflora of the gut (57,58,71,80). The present study
demonstrates alterations in intestinal cell mitotic and migration rates
when bacteria are introduced to animals previously in a germfree environment.
Intestinal bacteria or, alternatively, products of the bacteria may directly
affect small intestinal epithelial cell longevity on the villus and the
maturity of the enzyme systems. But it is likely the interaction of the
bacteria with the gut which causes these changes in the conventional animal,

for germfree animals receive food which is autoclaved and contains inert bacteria and bacterial products.

Factors other than bacteria, however, may affect intestinal cell population kinetics. Hypophysectomy and thyroidectomy (86) decrease intestinal cell mitotic and migration rates. The increase in cell proliferation following massive small bowel resection is thought to be mediated by hormonal factors (101). Since the same changes occur in the normal animal which is parabiosed to one which has previously undergone a significant resection of small bowel, humoral factors may indeed regulate intestinal cellular kinetics (102). Hormonal factors could also play a role in alterations induced after conventionalization.

The role of the normal intestinal bacteria in affecting intestinal epithelial cell turnover and inflammatory responses in the lamina propria, and conversely, the effect of the secretions of the gut on the intestinal microflora need further elucidation. The lamina propria of the germfree animal has few inflammatory cells and low circulating immunoglobulin levels. Introduction of bacteria increases both of these, probably as a result of antigenic stimuli from bacteria or bacterial products. The histology of the normal small intestine thus represents a response to a "chronic infection" in contrast to the germfree state. Further alterations in epithelial cell kinetics and increases in inflammatory cells occur in conventionalized animals after enteric infection. This situation may also pertain in the human in conditions said to be associated with greater numbers of bacteria in the proximal bowel and may account for the differences in small intestinal morphology encountered in various areas of the world (24,81,94,147).

Summary

The relationship between intestinal colonization and the small bowel mucosal cellular proliferation rate during conventionalization of the germfree mouse was examined. Sixteen mice were maintained under standard germfree conditions and fifty-four others were conventionalized. Migration of the small bowel epithelial cells was followed by radioautography with tritiated thymidine administration. Colonization was followed by qualitative and quantitative bacteriological fecal analyses. The percentages of the villi labeled (as determined by cell count) twenty-four, forty-eight and seventy-two hours following the thymidine administration showed immediate progression in the conventionalized animals from the germfree villus migration time (4 days) toward the conventional villus migration time (2 days). The epithelial migration rate of animals conventionalized for eight days was comparable to that of conventional animals.

A temporally related rapid progression of intestinal colonization was observed in the conventionalized animals. A stable bacterial pattern was cultured in the stool by the third day of conventionalization and by the seventh day it remained qualitatively and quantitatively similar to that of true conventional mice.

An adaptive balance between cell proliferation and sloughing, and thus migration rate, begins immediately with intestinal colonization of germfree animals and results in a doubling of the mucosal turnover after eight days, at which time both the intestinal migration rate and intestinal microflora are similar to that of conventional animals.

TABLE I

Schedule of injections and sacrifices in conventionalized and germfree animals.

TABLE I

SCHEDULE OF INJECTIONS AND SACRIFICES

CONVENTIONALIZED		GERMFREE	
INJECTIONS	SACRIFICES	INJECTIONS	SACRIFICES
1,2,3,4,5,6		1-16	
7,8,9,10,11,12	1,2		1-3
13,14,15,16,17,18,19,20	3,4,7,8		4-7
21,22,23,24,25,26,27,28	5,9,10,13,14,15		8-12
29,30,31,32,33,34	6,11,16,17,18,21,22,23		13-16
35,36,37*,38,39,40	12,19,24,25,26,29,30		
41,42,43,44,45	20,27,31,32,35,36		
46,47,48,49,50	28,33,34,37*,38,39,41,42		
51,52,53,54	40,43,44,46,47		
	45,48,49,51,52		
	50,53,54		

imal 37 died 1 day after conventionalization

TABLE II

Results of quantitative and qualitative stool cultures in germ-
free, conventionalized, and conventional mice. Stools were
positive for both aerobes and anaerobes on the first and second
days after conventionalization, but no counts were performed.
Selective culture media were also employed for Staphylococci,
Veillonella, and Diphtheroids, but none were recovered in any
of the stools cultured.

<div align="center">

TABLE II

</div>

|	Germfree Mice	Days After Conventionalization					Conventional Mice
		3	4	5	6	7	
Total Aerobes	no growth	3.0×10^7	1.1×10^8	3.2×10^7	4.9×10^7	3.7×10^8	8.0×10^7
Total Anaerobes	no growth	2.2×10^8	1.0×10^9	1.4×10^9	1.9×10^8	1.0×10^9	1.2×10^9
Coliforms		5.4×10^9	8.9×10^7	4.1×10^7	7.7×10^6	7.0×10^6	1.0×10^6
Streptococci		4.1×10^7	2.0×10^8	4.0×10^6	1.3×10^7	$<1.0 \times 10^4$	$<1.0 \times 10^4$
Lactobacilli		5.0×10^7	1.1×10^8	5.3×10^8	3.3×10^7	4.0×10^8	3.5×10^8
Bacteroides		$<1.0 \times 10^4$	$<1.0 \times 10^4$	6.0×10^8	1.0×10^8	2.0×10^5	1.3×10^8
Clostridia		2.0×10^4	1.7×10^8	$<1.0 \times 10^4$	$<1.0 \times 10^4$	$<1.0 \times 10^4$	$<1.0 \times 10^4$
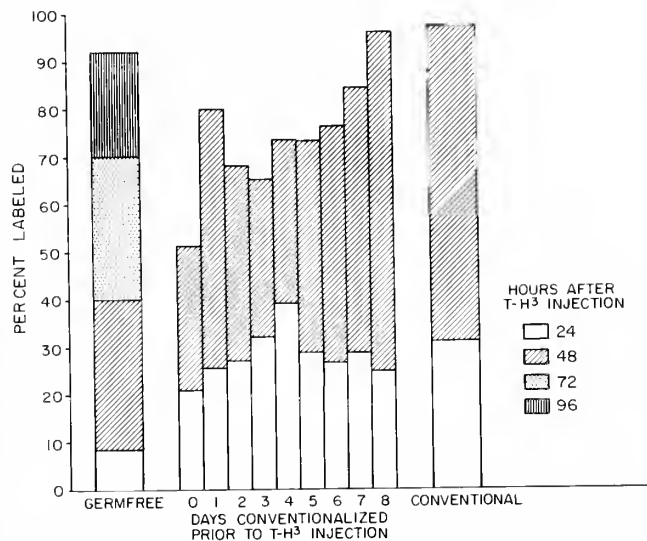
FIGURE 1: Percent of villi labeled (number of labeled cells divided by total number of cells x 100) after thymidine-H_3 injection in germ-free, conventionalized, and conventional mice. Values for conventional-ized animals at 72 hours were also determined and all showed 100% label-ling.
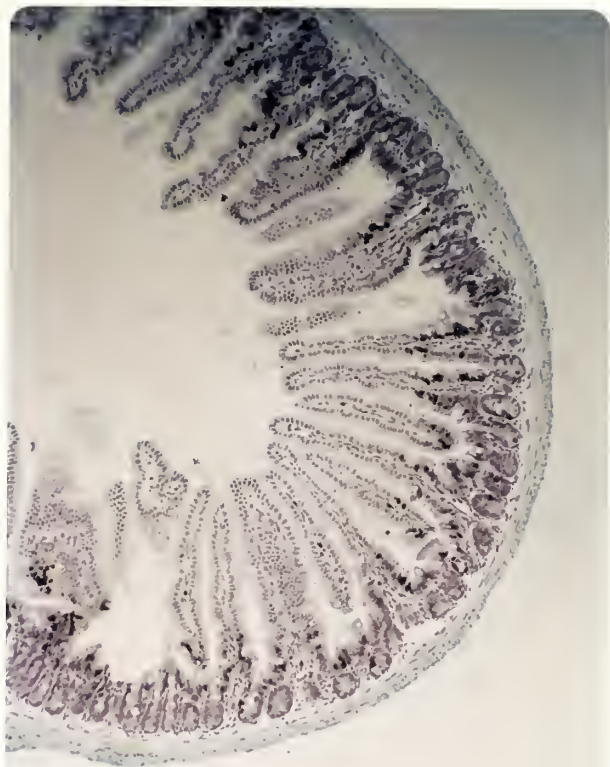
FIGURE 2: Radioautography in germfree mice after thymidine-H_3 injection.

        (a)   24 hours after injection
        (b)   48 hours after injection
        (c)   72 hours after injection
        (d)   96 hours after injection

The labeled column approaches total villus height after 4 days. Hematoxylin-light green x 100.
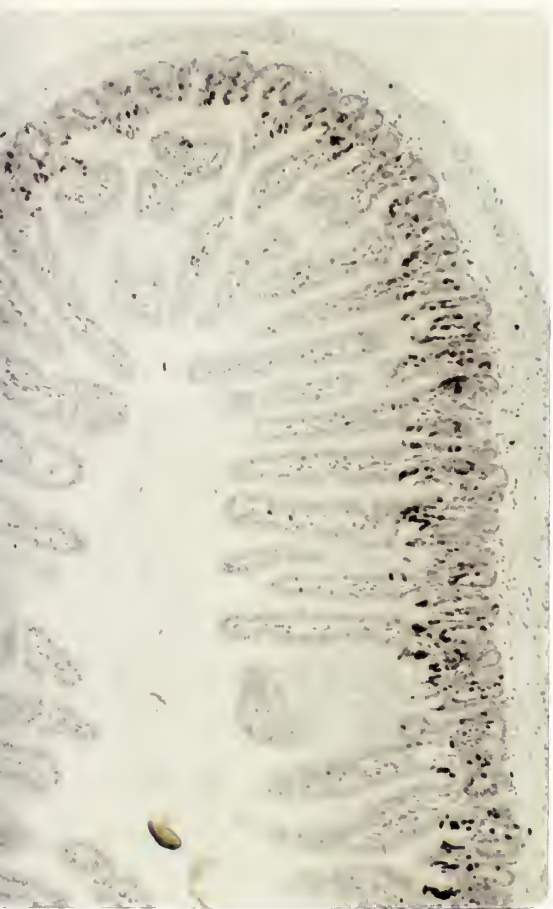
FIGURE 3: Radioautography in conventional mice after thymidine-H_3 injection.

       (a)   24 hours after injection
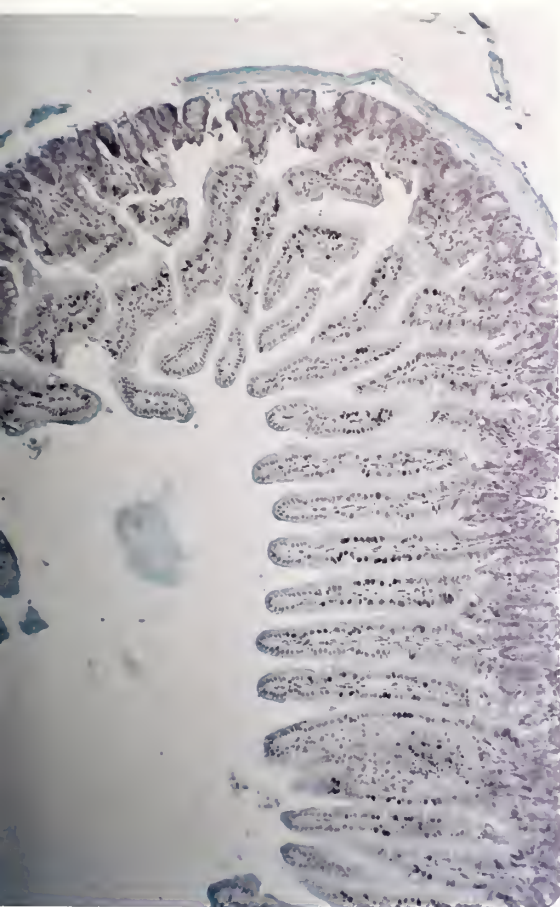   (b)   48 hours after injection

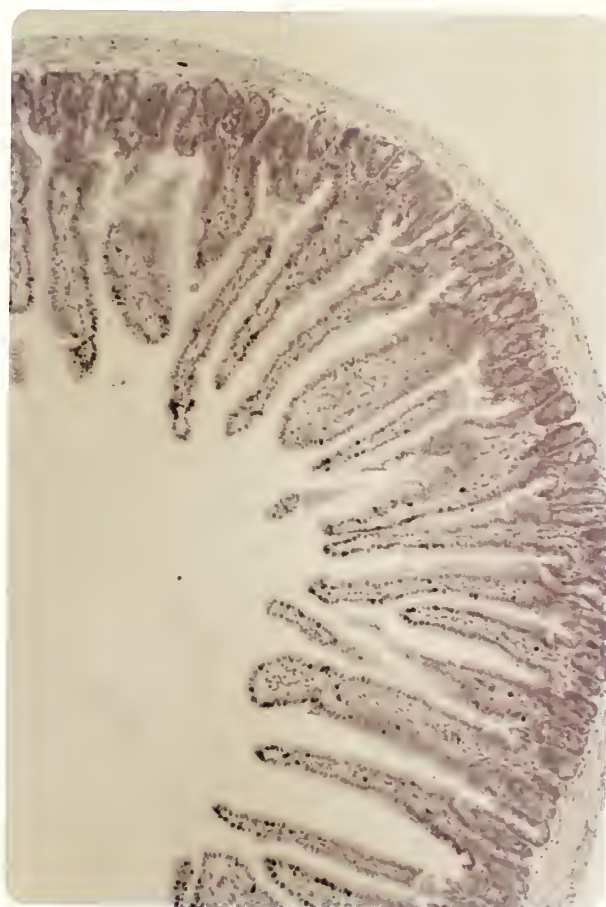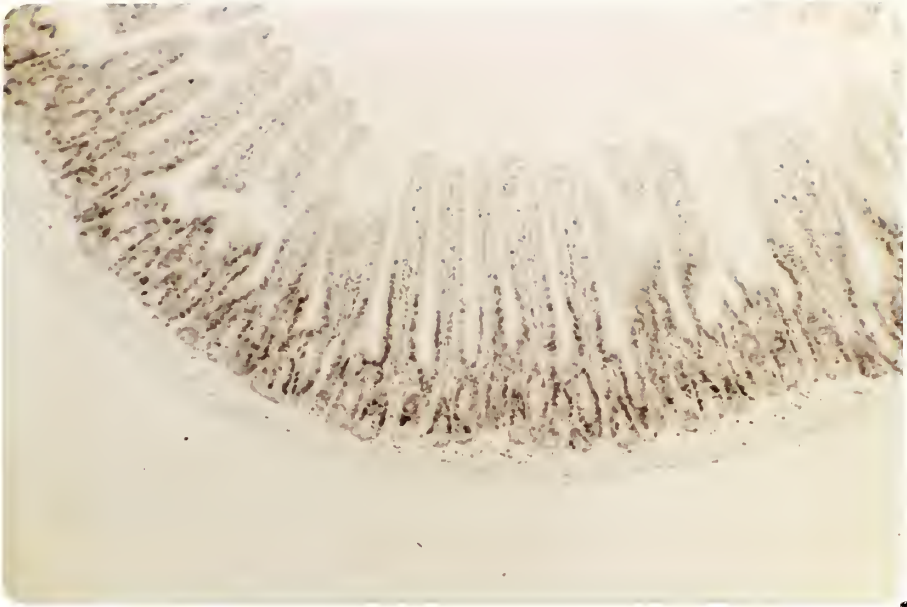The labeled column approaches total villus height after 2 days. Hematoxylin-light green x 100.

a
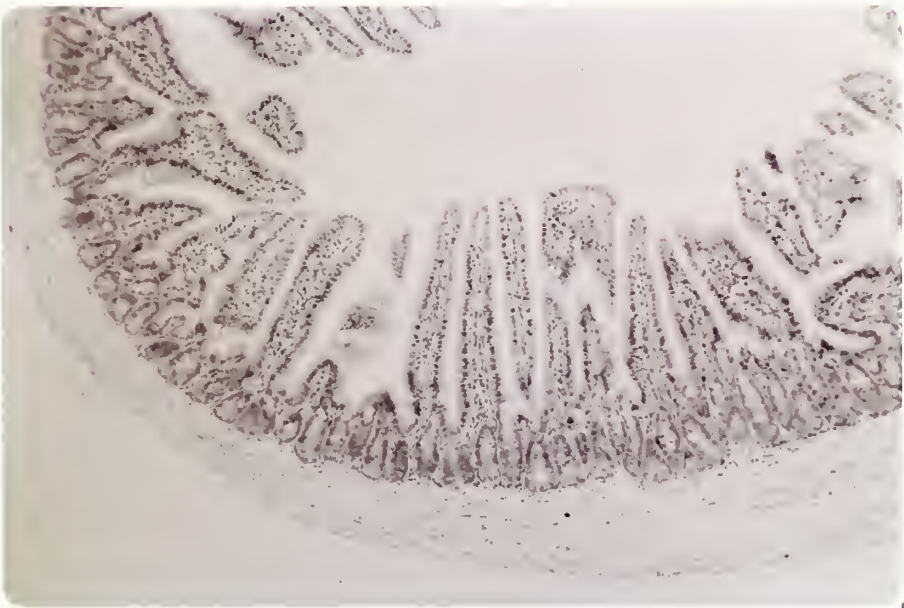
b

FIGURE 4: Radioautography in conventionalized mice after thymidine-H_3 injection.

(a,b) conventionalized 0 days prior to injection. x 100.
(c,d) conventionalized 2 days prior to injection. x 100.
(e,f) conventionalized 4 days prior to injection. x 100.
(g,h) conventionalized 6 days prior to injection. x 100.
(i,j) conventionalized 8 days prior to injection. x 100.

(a,c,e,g,i)   24 hours after injection
(b,d,f,h,j)   48 hours after injection

Hematoxylin-light green.

i

j
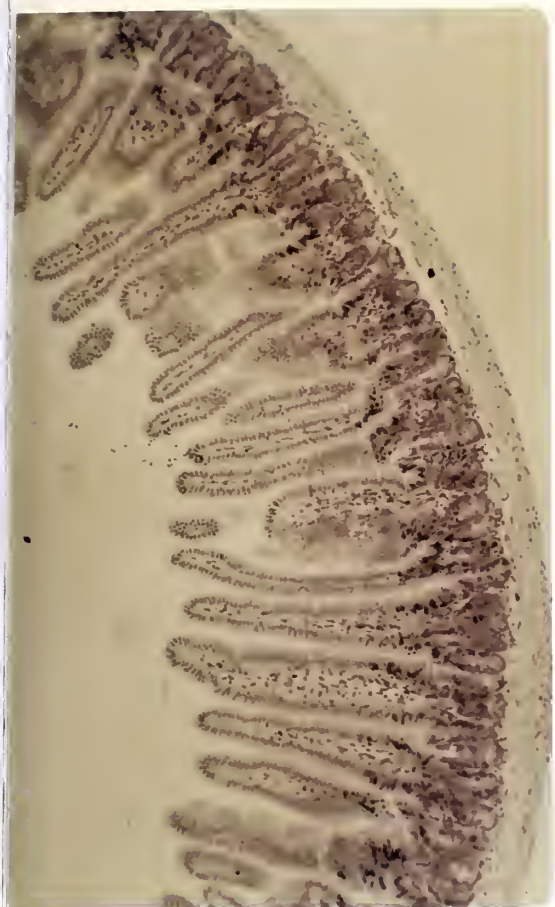
EFFECTS OF NEOMYCIN AND PENICILLIN ADMINISTRATION
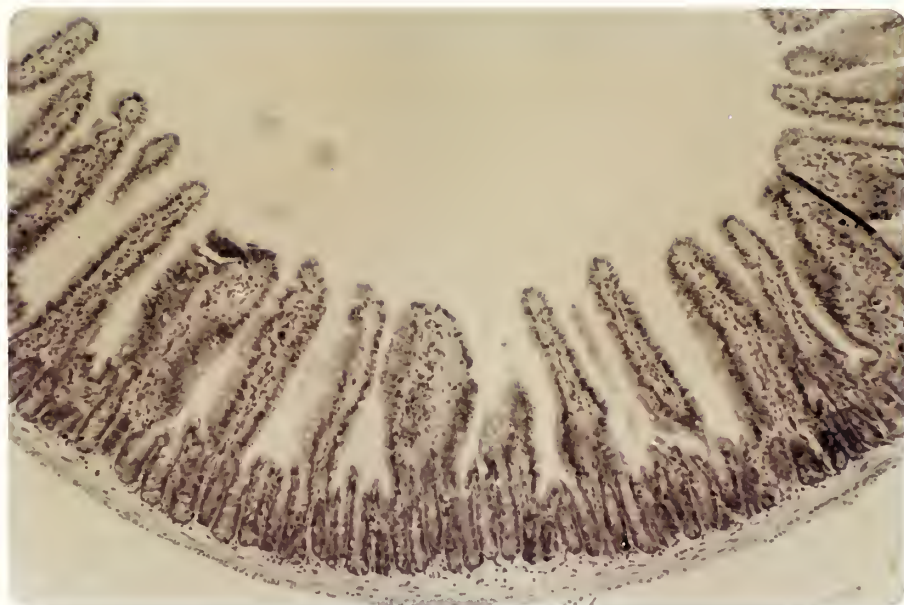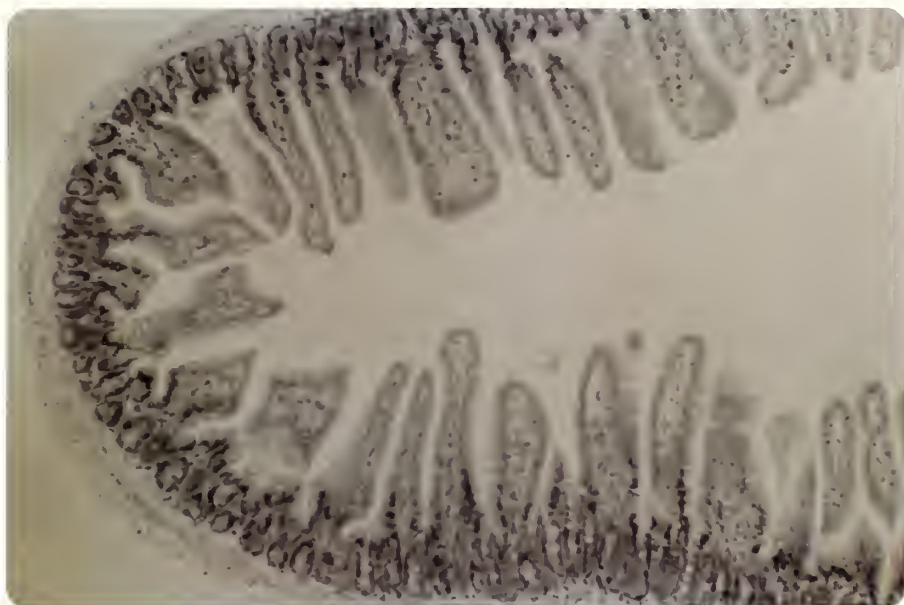ON MUCOSAL PROLIFERATION OF THE MOUSE SMALL INTESTINE:

Morphological and Function Correlations

The administration of the antibiotic neomycin has been associated with a reversible intestinal malabsorption syndrome (68,63,47,48,60); shorter, broader villi and decreased levels of intestinal enzymes have been reported both in humans (73,126,124,127,114,34) and in experimental animals (125,143,35). The severity of the intestinal morphologic and physiologic alterations appears to increase with dose and time (14,15,79).

Neomycin also reduces the quantity of intestinal bacteria, the intestinal environment becoming analogous to that in the germfree animal. The morphology of the gut of the germfree animal also differs from that of the conventional animal (58,2,65,80), but with longer, thinner villi and enhanced intestinal absorption of monosaccharides and amino acids (64,66). The absence of bacterial flora may account for the differences in gastrointestinal structure and function of the germfree animal from that of its conventional counterpart. Neomycin, on the other hand, may directly affect the epithelial cells of the small bowel, counteracting any villus alteration or enhanced absorptive capacity secondary to microflora reduction.

The present study was designed to assess the effect of an antibiotic regimen on the fecal microflora and on small intestinal function and morphology. Mice were fed neomycin and penicillin with evaluation of small intestinal cell population kinetics, intestinal transport of an amino acid, and intestinal enzymes by disaccharidase assay of the brush border and histochemical staining reactions of epithelial cell enzyme systems.

Materials and Methods

Intestinal Transport:

Sixteen male and sixteen female CFW mice[+], 70 days old and with an average weight of 27 gms, were randomly divided into two groups of 8 males and 8 females each. All mice were fed a standard animal diet[*] which does not contain antibiotics; food and water were allowed ad libitum. Mice were housed on sanicil bedding in plastic cages in a conventional animal room. One group received an oral antibiotic regimen daily while the second served as a control. Potassium penicillin G[++] and neomycin sulfate (mycifradin sulfate[**]) were dissolved in distilled water, 2.4 gms (4 million units) and 10 gms respectively per liter. Fresh antibiotic solution was prepared twice a week and kept at 4°C until it was administered. Animals were fed the antibiotic solution as drinking water. Consuming, on the average, 5 ml of water per day, each mouse received 50 mgms of neomycin and 12.0 mgms (20,000 units) of penicillin per day. The control mice were on tap water. Weight of a constant sampling of both the control and the antibiotic-treated mice was recorded twice weekly.

Three control and three experimental mice were sacrificed by a snap of the neck 8, 16, 22, 29, and 36 days after the initiation of the antibiotic regimen. Intestinal transport of an amino acid was evaluated by the everted gut sac technique (156). Immediately after sacrifice, the small intestine was isolated, stripped of its mesentery and rinsed with

+ Carworth Farms, New City, New York.
* Purina Lab Chow, Special Formula 5010C, Purina Labs., St. Louis, Mo.
++ E.R. Squibb and Sons, New York.
** Mycifradin sulfate, 60% as neomycin base, Upjohn, Kalamazoo, Michigan; 11.35 mM (700mg%) neomycin used.

Krebs-bicarbonate buffer. The intestine was everted and then divided into three approximately equal segments. Crystals of 1-methionine-methyl-C^{14} were dissolved in Krebs buffer to a concentration of 1×10^{-3} and 1.0 ml was introduced into each everted sac. Each sac was subsequently placed in an Erlenmeyer flask containing 5.0 ml of buffer with an identical concentration of the labeled methionine as that instilled on the mucosal side. The flasks were gassed with a mixture of 95% oxygen -5% carbon dioxide and incubated in a Dubnoff shaker at 37°C for 1 hour. At the end of this period, each sac was drained, the volume was recorded and each segment was then weighed. Aliquots of the mucosal and serosal fluids were analyzed for radioactivity in a liquid scintillation counter[++]. Results were calculated and expressed as micromoles of methionine transported per gram wet tissue weight of intestine per hour.

Radioautography:

Small intestinal cell proliferation kinetics were studied in 45 antibiotic-treated mice and 16 control mice of the same strain[**]. Thirty microcuries of sterile thymidine-methyl-H^{3}[*] (6.7 curies/millimoles in 0.3 ml sterile water) was injected intraperitoneally. Groups of nine antibiotic-treated animals were injected at weekly intervals up to five weeks after initiation of treatment. Eight control animals were injected after one week and the remaining eight on the fifth week of the experiment, at the same times as the treated mice.

[++] Packard Tri-Carb Liquid Scintillation Spectrometer.
[**] CFW mice, Carworth Famrs, New City, New York.
[*] New England Nuclear Corporation, Boston, Mass.

Segments of the small bowel were taken 5 cm proximal to the ileocecal junction as the mice were sacrificed under ether anesthesia according to the schedule outlined in Table 1.

The intestinal specimens were then treated as described on page six.

Bacteriology:

Qualitative and quantitative bacteriologic cultures of the stools of three randomly selected control mice and of three randomly selected experimental mice, prior to initiation of treatment, were performed on selective media. 0.1 gm of the stool sample was treated as described on page seven. Thereafter, fecal cultures were performed prior to the time of sacrifice on all the antibiotic-treated animals used in the transport studies. The first transport control group, sacrificed on the eighth day of the experiment, was similarly cultured. Ceca of the control and of the antibiotic-treated mice were compared for size at time of sacrifice.

Histochemistry:

Enzymes of the small intestine brush border and epithelial cells were evaluated by histochemical staining techniques (53). Eight control and 45 mice treated with antibiotics for one to five weeks were anesthetized with ether, after which two segments of the proximal bowel (about 5 cm from the pylorus) and two segments of the distal bowel (about 4 cm from the ileocecal junction) were removed and rinsed with Krebs buffer. A portion of the left lobe of the liver was also removed. Sections of gut from each animal were placed on the portion of that animal's liver, which served not only as support of the intestinal sample but also as control for the enzymes studied. The specimens were immediately immersed in liquid nitrogen, and stored at -70°C in an airtight polyethylene bag.

Histochemical reactions were carried out on cryostat sections cut 4 microns thick at -20°C and picked up on a cover slip. The following enzymes were studied: acid and alkaline phosphatase, non-specific esterase, NADH dehydrogenase, NADPH dehydrogenase, lactic dehydrogenase, and succinic dehydrogenase. The histochemical reactions were performed simultaneously on both control and experimental animals and then compared for the enzymes studied. The slides were coded and the intensities of the stain were evaluated on a 0 to 5 scale by 2 investigators and one technician.

Disaccharidase Assay:

At the time of obtaining tissue for the histochemical studies, adjacent portions of the proximal gut were also obtained for assay of disaccharidases. The small intestine was cut open and the mucosa scrapped off with a glass slide onto a piece of previously weighed cork. The specimen was weighed and then homogenized with four times its weight of distilled water. Assays were performed for lactase and sucrase content by the Dahlqvist method (29). A group of eight germfree mice* of the same strain was similarly studied. Results were recorded as micromoles disaccharide hydrolyzed/min/gm of wet tissue weight.

*Carworth Farms, New City, New York.

Results

Transport:

Total transport of l-methionine in the antibiotic-treated animals
$(1.6 \pm 1.2')^*$ did not differ signficantly when compared to that of the
control mice (1.8 ± 1.0) (p <.25). When transport in all the three seg-
ments of intestine was compared for each week studied, the only signifi-
cant difference found was after the fourth week of therapy. At this time,
the transport in the experimental group was reduced (0.7 ± 0.5) compared
to the control (1.7 ± 0.6) (P <.005). Methionine transport was also cal-
culated for each third of gut studied (Table II). Only the distal third
of the gut of the experimental group transported less amino acid than
that of the control group.

Radioautography:

After the first and second weeks of antibiotic therapy, there were no
significant differences in intestinal cell proliferation between the experi-
mental and the control groups. Cell counts done twenty-four hours after
the injection of labeled thymidine in animals on the antibiotic regimen
for three, four, and five weeks revealed a significant increase in the
height of labeled cells (Table III); that is, more epithelial cells were
labeled in the villi of the antibiotic-treated group $(35.0 \pm 7.0$ to 23.8
± 4.3 in controls, Figure 1). When the results were separated by the
animals' sex, it was observed that the treated males had a greater number

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' Standard deviation.

* Values expressed as micromoles of methionine transported per gm wet
tissue weight per hour.

of cells labeled than the treated females (37.9 ± 9.8 to 32.1 ± 1.8),
and likewise each group had more than their corresponding controls
(24.2 ± 6.0 and 23.4 ± 2.8 respectively). There was a greater variance,
however, in the number of labeled cells in the males (Figure 2). A
smaller increase in the total number of epithelial cells in the villi of
the antibiotic-treated animals was present compared to the controls
(63.8 ± 3.4 to 57.6 ± 5.5). Therefore, the percent of labeled cells
(number over the total cells) was greater for the antibiotic group three
to five weeks after therapy than for the controls (55.4 ± 13.9 to 41.5 ±
6.9) (Figure 3 and 4).

Only the sections taken at 24 hours after injection of the labeled
thymidine were counted for comparative purposes, since in those taken 48
hours and thereafter in both groups all the epithelial cells in the villi
were labeled. The total number of epithelial cells per villus, however,
was again significantly greater in the experimental group.

Bacteriological Flora:

The quantitative and qualitative bacterial recoveries were similar in
the two groups at the start of the experiment. Eight days after antibiotic
therapy and thereafter only yeast organisms were recovered from the stools
with the culture media employed (Table IV).

Enlarged ceca developed by the first week of antibacterial treatment
and the ceca remained enlarged for the remaining four weeks (Figure 5).
Cecal contents were fluid. Watery stools, similar to those seen in the
germfree state, also developed by one week of treatment and persisted
throughout the experiment.

Histochemistry:

Histochemical assay of non-specific esterase and NADH dehydrogenase
levels in the proximal gut showed decrease in staining reaction in the
antibiotic-treated mice when compared to control mice (Figure 6). These
differences were not apparent in the specimens of the distal gut. Stains
for acid and alklaline phosphatase, NADPH dehydrogenase, lactic dehydro-
genase and succinic dehydrogenase in both proximal and distal gut were
similar in both groups of animals (Figure 7).

Lactase and Sucrase Activity:

The activity of sucrase of the antibiotic-treated mice $(11.4 \pm 1.3')^*$
was slightly increased as compared to that of the controls (9.7 ± 2.8)
$(p < .1)$. Lactase activity $(1.2 \pm .4)$ also was not significantly decreased
when compared to the controls (1.5 ± 4) $(p < .25)$. A reduction of the
lactase levels was more apparent after 16 days of treatment $(.8 \pm .3)$
$(p < .1)$.

In the germfree mice, however, significantly increased levels of
sucrase (13.1 ± 2.6) were present compared to their controls (8.0 ± 2.3)
$(p. < .05)$, while lactase activity was only slightly higher $(1.6 \pm .4$ com-
pared to 0.9 ± 0.7 in the controls) $(p < 0.1)$.

Weight:

At the onset of the experiment, the control mice weighed 27.5 ± 2.9 gm
which increased to 30.5 ± 2.0 gm over the 35 day period. Antibiotic-treated

---

' S.D.
* Values expressed as micromoles of disaccharide hydrolyzed/min/gm.

was slightly distorted

mice went from a weight of 27.0 ± 4.8 gm to 31.5 ± 4.2 gm over the same period. The experimental animals thus gained weight comparably to the control group.

Discussion

In the present study, the oral administration of neomycin and penicillin was associated with a reduction in the intestinal bacterial flora. Neomycin also may have direct adverse effects on the gut, accounting for the observed alterations in the small intestine.

Neomycin may cause a malabsorption syndrome (68,63,47,48,60). In humans, as with the animals, this reversible effect increases with time and dose (14,15,79). Light and electron microscopic changes in the crypt cells, blunting of villi and inflammatory cell infiltration of the lamina propria have been reported (34,21). Loss of microvilli may also be present (21). In humans, neomycin induced steatorrhea is more severe when the drug is given orally or when instilled directly into the jejunum than when instilled directly into the lower bowel (60). The magnitude of the observed azotorrhea, however, is the same whether the drug is given orally or instilled directly into jejunum or ileum (60). In addition to its adverse effects on the small intestine, neomycin has been shown to inhibit lipolysis in vitro (107) and to bind bile salts by forming a precipitate at both the bile salt concentrations and the pH usually found in the duodenum (47,48, 127). The administration of bile salts and pancreatic enzymes concurrent with the antibiotic does not consistently ameliorate the steatorrhea of neomycin malabsorption (60). Thus the steatorrhea of neomycin therapy in humans appears to be a combination of maldigestion and malabsorption.

In rats treated with large doses of neomycin, an apparent increase in the uptake of 1-phenylalanine by ileal slices may have been related to the associated starvation described in these animals (125,157). In another study, this antibiotic depressed the absorption of glucose in monkeys with Thiry-Vella loops of mid small intestine (54). The time and dose dependent effect of neomycin on absorption occurred in the absence of morphologic alterations (54). High doses of neomycin in rats have also caused an impairment in the absorption of carbohydrates, also without changes in the intestine (143,15). The only change in transport of methionine occurred in the animals treated for four weeks; in all the treated animals, only the distal segments of gut showed this decrease. On the other hand, in some species (67) diets containing other antibiotics as penicillin or tetracycline have been followed by an enhanced absorption of the nutrient tested (67,38,6). Excess weight gain has been described in some of these animals (40). This improved growth may be related to alterations in the intestinal bacterial flora, concomitant with an enhanced intestinal absorption of nutrients. In our study, however, no significant differences in weight were noted.

The intestinal absorption of monosaccharides and of amino acids is greater in germfree animals than in their conventional counterparts (65, 64,66). Monocontamination of germfree animals decreases this absorptive capacity (66). In addition, conventionalizing germfree animals induces changes in small intestinal morphology, altering the height and width of villi and increasing inflammatory cells in the lamina propria (65,80,66). The antibiotic regimen used in the present study removed recoverable organisms from the gut, except for yeast. The gross morphologic findings of thin intestinal walls, enlarged ceca, and watery stools in the treated

mice are also encountered in animals reared in germfree environments (58, 80,134,22,137). The administration of various antibiotics to adult mice (42) has also induced enlarged ceca with fluid contents within a day of antibacterial therapy (134). Alterations in the mechanisms of water transport may result from the changes in the intestinal microflora, as the aforementioned characteristics occur in both germfree and antibiotic-treated animals. Differences from the usual microbial flora occurred with each antibiotic employed, but after two days a secondary micro-flora developed in all the treated animals (134). The absence of intes-tinal bacteria in our mice is related to the administration of the two antibiotics.

In some studies, decreased levels of various intestinal enzymes following neomycin have been reported (79); in other studies no such alterations have been noted (54,119). In man, histochemical studies have demonstrated decreased staining reactions of succinic dehydrogenase and ATPase (79). Histochemical evaluation of six enzymes were not altered, however, in monkeys with Thiry-Vella fistula (54). Non-specific esterase and NADH dehydrogenase staining reactions were decreased in our treated mice, compatible with direct injury of the intestinal epithelial cells. This finding is also compatible with the observed increase in the rate of cellular proliferation, for less mature intestinal epithelial cells may have less developed enzyme systems (113). More mature epithelial cells on villi where lower rates of cell migration are present, as in germfree animals, may have well developed enzyme systems, as suggested by stronger histo-chemical staining reactions (113).

Disaccharidase activity in the brush border of the small intestine is decreased both in association with neomycin therapy (124,114) and in intestinal malabsorptive syndromes (116,31). In humans, disaccharidases were reduced after three days of treatment with a daily dose of 8 gm of neomycin (21). In the present study, lactase activity in the antibiotic-treated animals was diminished, a finding compatible with the observed high cellular proliferation rate. The diminution of lactase, an enzyme which is sensitive to intestinal injury, could also reflect a direct toxic effect of neomycin on the gut. Sucrase levels, however, tended to be higher in the treated than in the controls. These changes are not reflections of adaptations to the diet since both groups received the same diet.

In one study, neomycin was reported to cause an increase in mitotic count, explained as mitotic arrest of undifferentiated crypt cells (34). The present study suggests that this antibiotic increases cell proliferation and migration rates. Despite the absence of intestinal micro-organisms, except for yeast, the mitotic and migration rates did not resemble those reported for germfree animals, but rather approximated the changes observed after enteric infections (1). It is suggested that neomycin induces these changes in dynamic intestinal morphology independent of the reduction of microflora. The effect may be mediated by a hormonal factor. This seems to be the case in massive small bowel resection on the basis of parabiotic observations (102). Hormonal effects may also explain the differences in cellular kinetics observed between the males and females.

The cell proliferation rate of the mucosa of the small bowel and the migration rate of the epithelial cells of the villi may be viewed as a spectrum. On the one end is the low rate of the germfree state, producing

very mature cells on the villi, which appear to contain greater enzymatic activity (119), and whose intestine has an enhanced transport capacity (66). At the other end is the inflammed gut with a high cellular proliferation rate (1,8,140) and young cells with less enzymatic activity. In these animals, absorptive capacity may be reduced. The conventional animal may thus represent the middle portion of this spectrum. Grossly, the gut of antibiotic-treated animals may resemble that of germfree animals; however, the direct effects of neomycin on the intestine may cancel whatever effect the reduction of the intestinal microflora may have on morphology and function of the small intestine.

Summary

The effects of an oral neomycin and penicillin regimen on intestinal bacteriology and on morphology and function of the small intestine of mice were investigated. Quantitative and qualitative stool cultures on selective media of the treated animals revealed only growth of yeast organisms. The treated animals developed enlargement of the ceca with fluid contents and watery stools, resembling characteristics of germfree animals.

Radioautography with tritiated thymidine revealed an increased epithelial cell migration rate in the mice treated with the antibiotics for three to five weeks. A slight increase in villus height was also noted. The treated male mice showed greater variance than the treated females in epithelial cell migration rates.

Histochemical staining reactions showed a decrease in non-specific esterase and in NADH dehydrogenase activity in the proximal gut of the antibiotic animals. Stains of distal gut and those for acid and alkaline phosphatase, NADPH dehydrogenase, lactic dehydrogenase, and succinic dehydrogenase were similar to the controls. A slight increase in sucrase activity and a slight decrease in lactase activity in the antibiotic animals was observed in contrast to control animals. Germfree mice, however, had greater sucrase and lactase activity. Transport of 1-methionine was slightly reduced in the distal segment of the treated animals.

Since the direction of these changes is away from the intestinal state observed in germfree animals, they are likely the result of the direct action of the antibiotics on the gut.

TABLE I

Schedule of sacrifices following thymidine-H^3 injection.

TABLE I

Hours after injection of Thymidine-H^3	Number of Animals Studied	
	Control	Antibiotic
24	4	2
48	4	3
72		2
96		2

TABLE I

Hours after injection of Thymidine-H3	Number of Animals Showing	
	Control	Metastatic
24	4	
48	4	3
72		
96		

TABLE II

Transport of l-methionine by the proximal, middle, and distal
intestine in antibiotic-treated and control mice.

TABLE II

TRANSPORT IN LEVELS OF THE SMALL INTESTINE

Level of Small Intestine	Control	Antibiotic	$p^{\otimes}$
Proximal	$0.8 \pm .5^{\circledast}$	0.7 ± 0.7	$< .4$
Middle	$1.5 \pm .6$	1.4 ± 0.9	$< .4$
Distal	$2.7 \pm .7$	1.9 ± 1.2	$< .05$

$^{\otimes}$By Student's t-distribution

$^{\circledast}$Micromoles of methionine transported per gm wet tissue weight per hour $\pm$ S.D.

TABLE III

Radioautography in control and antibiotic-treated male and
female mice 24 hours after thymidine-H^3 injection.

TABLE III

RADIOAUTOGRAPHY IN CONTROL AND ANTIBIOTIC MICE
24 HOURS AFTER INJECTION OF LABELED THYMIDINE

SEX	MEASUREMENT	CONTROL	ANTIBIOTIC 1-5 WEEKS	P*	ANTIBIOTIC 3-5 WEEKS	P*
Both	No. of labeled cells/villus	23.8 ± 4.3	30.3 ± 9.7	<.05	35.0 ± 7.0	<.0025
	Total cells/villus	57.6 ± 5.5	63.5 ± 3.6	<.01	63.8 ± 3.4	<.025
	Percent	41.5 ± 6.9	48.2 ± 16.8	<.2	55.4 ± 13.9	<.025
♂	No. of labeled cells/villus	24.2 ± 6.0	28.9 ± 14.1	<.30	37.9 ± 9.8	<.05
	Total cells/villus	60.5 ± 2.4	63.8 ± 3.9	<.10	63.8 ± 4.1	<.15
	Percent	39.7 ± 8.4	45.8 ± 24.4	<.35	60.4 ± 19.8	<.10
♀	No. of labeled cells/villus	23.4 ± 2.8	31.9 ± 1.9	<.0005	32.1 ± 1.8	<.005
	Total cells/villus	54.8 ± 6.5	63.3 ± 3.7	<.025	63.8 ± 3.6	<.05
	Percent	43.2 ± 5.7	50.5 ± 5.4	<.05	50.4 ± 4.4	<.10

Percent = No. of labeled cells per villus divided by total no. of cells per villus X 100.

+ Standard Deviation
−

* By Student's t-distribution

TABLE IV

Results of quantitative and qualitative stool cultures in control and antibiotic-treated mice. Selective culture media were also employed for Staphylococci, Veillonella, Clostridia and Diphther-oids but none were recovered in any of the stools cultured.

TABLE IV

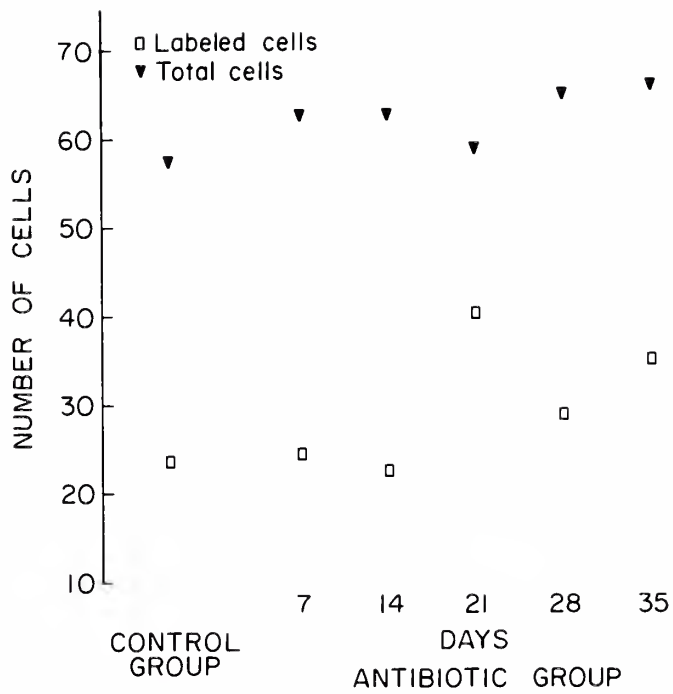|	DAYS ON ANTIBIOTICS: 0	0	8	8	16	22	29	36
STATE OF ANIMAL:	CONTROL	EXPERIMENTAL	CONTROL	EXPT.	EXPT.	EXPT.	EXPT.	EXPT.
Total Aerobes	1.1×10^6	7.9×10^6	5.0×10^7	–	Yeast	Yeast	–	Yeast
Total Anaerobes	2.0×10^9	2.7×10^8	3.7×10^8	–	–	Yeast	–	–
Coliforms	2.0×10^6	8.6×10^5	3.0×10^4					
Streptococci	1.0×10^8	4.0×10^7						
Lactobacilli	4.0×10^8	5.0×10^8	3.0×10^8					
Bacteroides	5.2×10^5	3.0×10^5	2.6×10^8					
Yeast			Yeast		2.0×10^7	5.0×10^5	1.4×10^5	4.0×10^5

FIGURE 1

Total and labeled cells of villi in all control and antibiotic-treated mice 24 hours after thymidine-H^3 injection.

△ Labeled cells ♂
▲ Total cells ♂
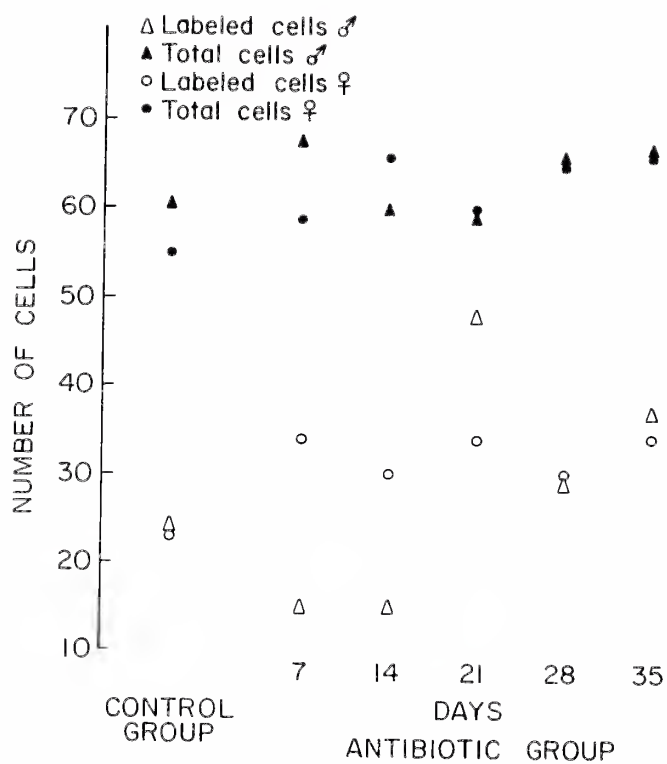○ Labeled cells ♀
● Total cells ♀

FIGURE 2

Total and labeled cells of villi in male and female control
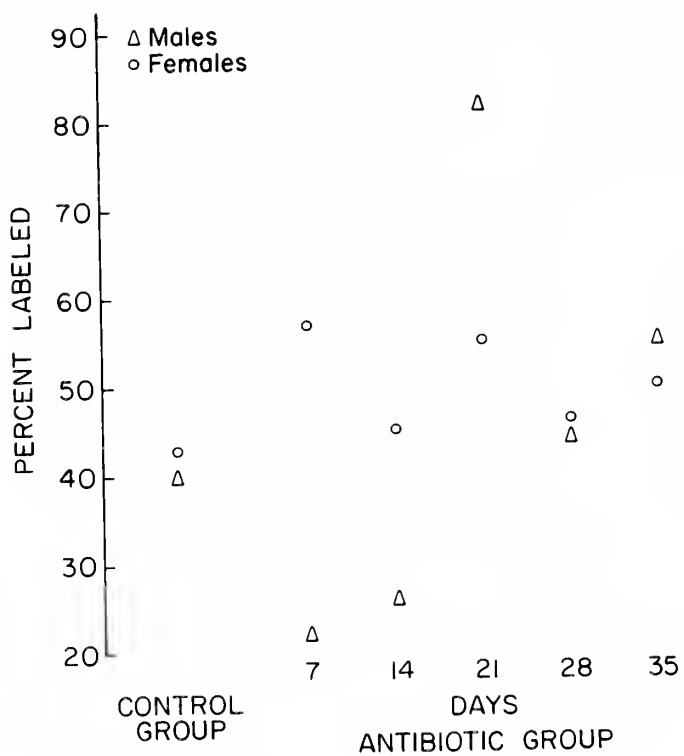and antibiotic-treated mice 24 hours after thymidine-H^3 injection.

FIGURE 3

Percent of villus labeled (number of labeled cells per villus divided by the total number of cells per villus x 100) in male and female control and antibiotic-treated mice 24 hours after thymidine-H^3 injection.
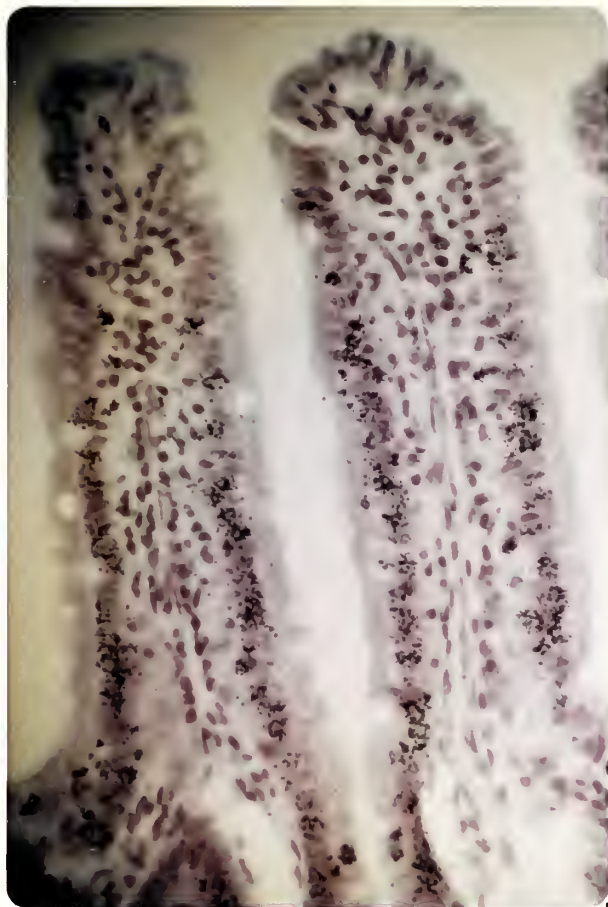
FIGURE 4

Radioautography in control and antibiotic-treated mice 24 hours
after thymidine-H^3 injection.

    (a)   female control
    (b)   female on antibiotics for 22 days
    (c)   male control
    (d)   male on antibiotics for 36 days

Note the greater height of the labeled column in the treated
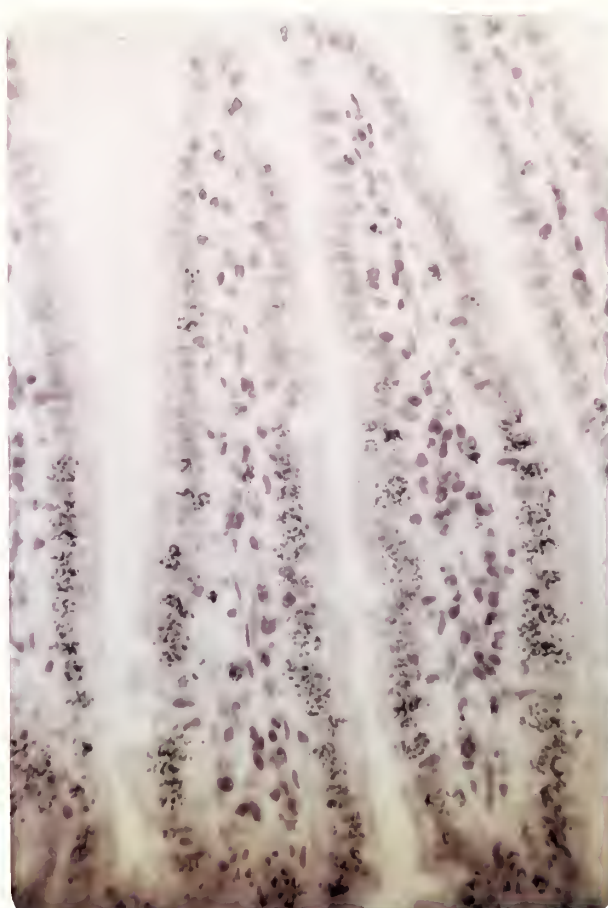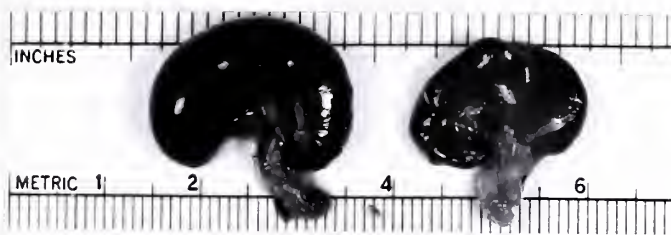animals. (Hematoxylin--light green x 400).
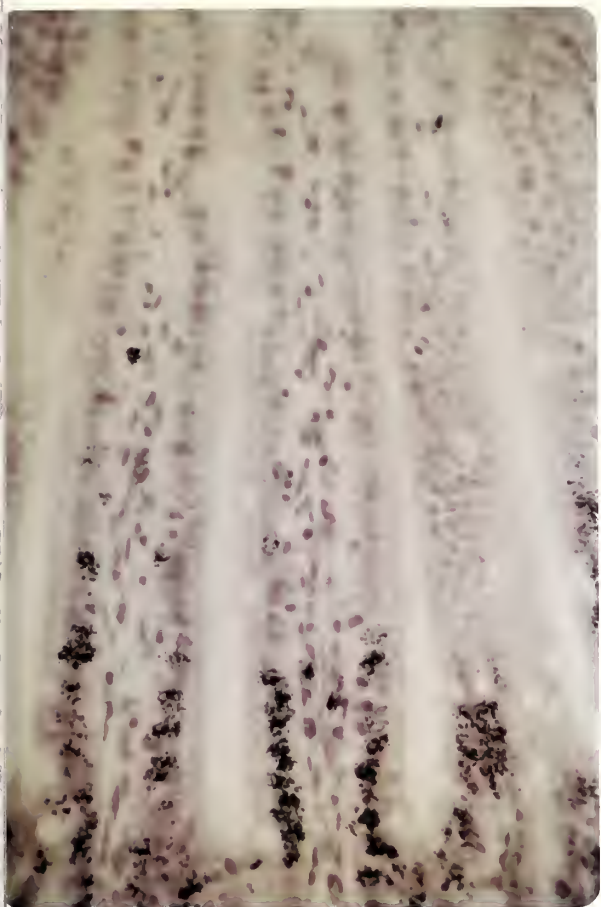
FIGURE 5

The 2 gm cecum on the left comprised 8% of the body weight of
a mouse on antibiotics for 38 days. The ceca of antibiotic-
treated mice consistently appeared 2 to 3 times larger than
the conventional mice ceca which, like the one on the right,
compromised 1-2% of the body weight.

FIGURE 6

Photomicrographs of the intestine of control and antibiotic-treated mice.

(a) NADH reaction, control
(b) NADH reaction, treated 30 days
(c) non-specific esterase reaction, control
(d) non-specific esterase reaction, treated 32 days

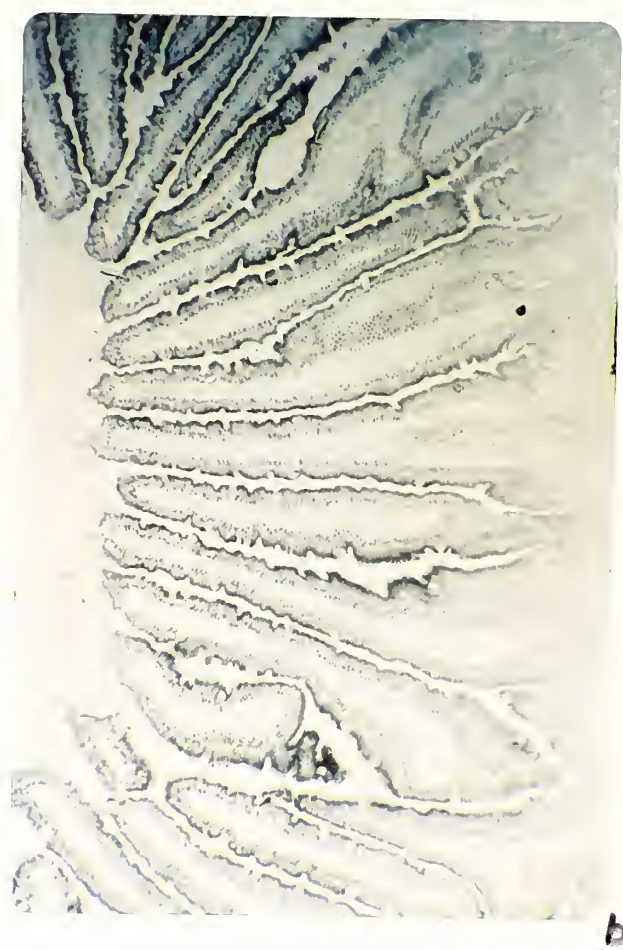Staining reactions are diminished in the treated animals (x 100).

FIGURE 7

Photomicrographs of examples of intestinal histochemistry of
control and antibiotic-treated mice.

    (a)   acid phosphatase reaction, treated 30 days
    (b)   alkaline phosphatase reaction, treated 30 days
    (c)   NADPH dehydrogenase reaction, treated 23 days
    (d)   lactic dehydrogenase reaction, control
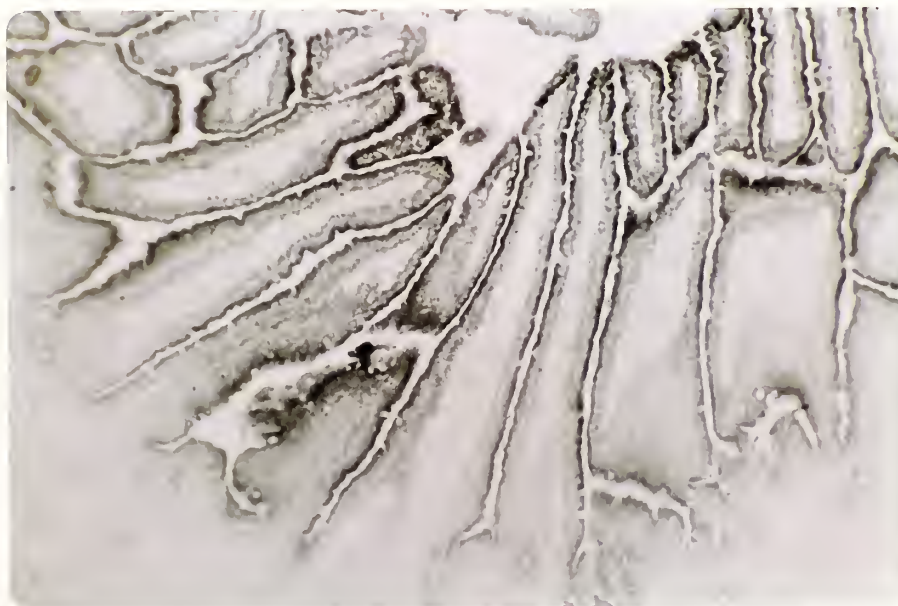   (e)   succinic dehydrogenase reaction, treated 37 days

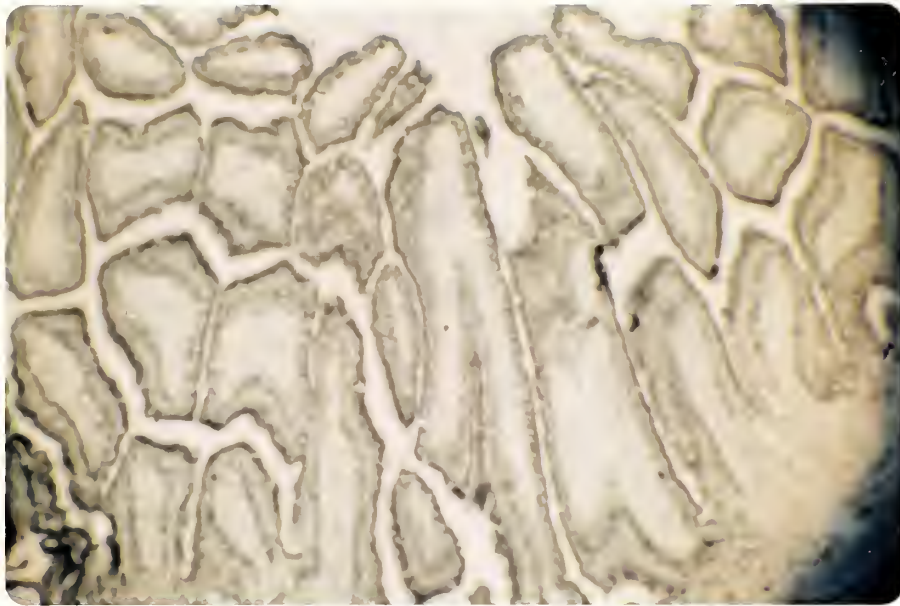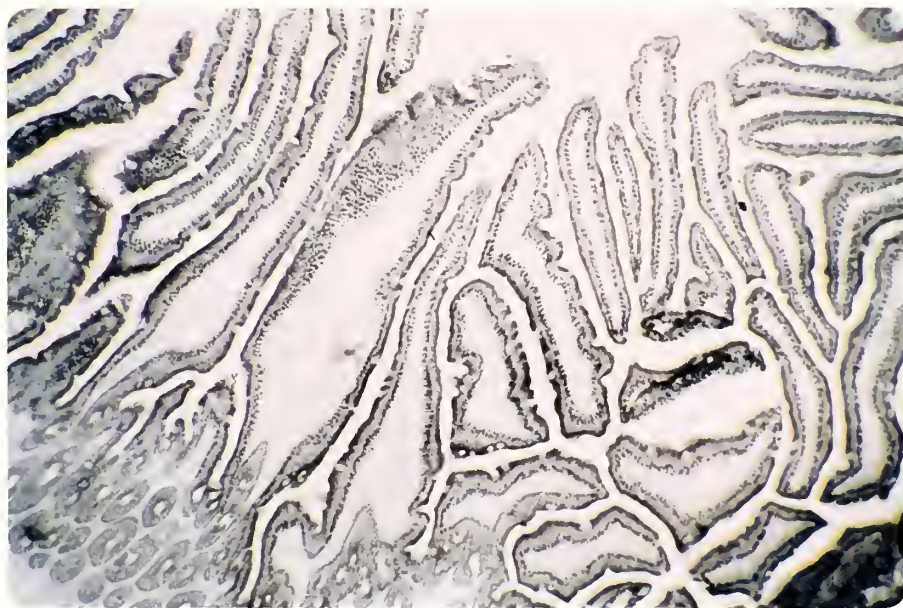Staining reactions were similar in control and treated animals
(x 100).

a
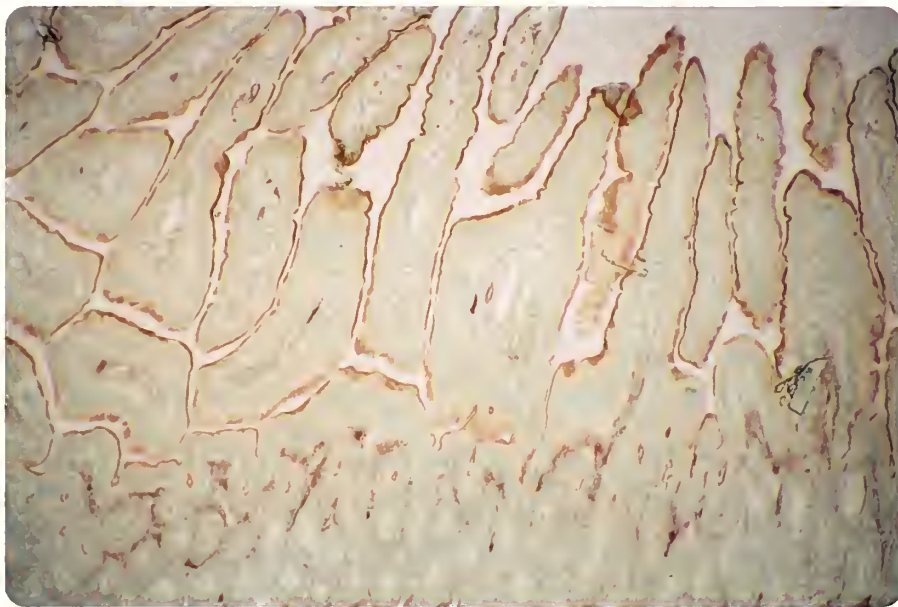
b

c

d

e

General Conclusions

The relationship between the intestine and its bacterial micro-
flora has been investigated, especially with regards to mucosal cell
proliferation. The unifying concept is one of a spectrum of small intes-
tinal mucosal proliferation, ranging from the four day migration time
in the germfree mouse villus to the two day migration time in the con-
ventional counterpart. Conventionalized mice display transitional mucosal
kinetics, moving from the germfree rate to the conventional rate over an
eight day period. Mice fed neomycin and penicillin possess a mucosal
proliferation rate significantly greater than conventional mice, compar-
able to animals with enteric infections, in which the inflammatory reaction
in the gut is extensive. The germfree mucosal cell proliferation rate
thus at present appears to be a baseline, although this may only be rela-
tive, since germfree animals are fed autoclaved diets containing dead
bacteria. In this light, the proliferation rate in conventional animals
may be regarded as one of physiological inflammation.

This is not, however, to say that because variations in cell prolifera-
tion are associated with variations in intestinal micro-organisms that
bacteria necessarily affect the mucosal cell proliferation directly. Hor-
mones may play a part in mucosal proliferation as they do in epidermal
cell proliferation (131). Nutritional deficiencies also significantly
alter cellular proliferation rates (70). Further experimentation may
delineate more precisely the specific influences of intestinal bacteria,
nutrition and other factors upon mucosal proliferation.

The significance of mucosal proliferation relates to intestinal function. Since it appears that villi with higher proliferation rates are lined by less "mature" epithelium than those with lower proliferation rates, the physiological performance of the gut in both digestion and absorption may be related to the mucosal proliferation rate. The function of the entire organism is then secondarily related. Differences in mucosal proliferation based on differences in intestinal colonization may account, along with climatic and genetic factors, for the variations observed in intestinal morphology throughout the world (24,81,94,147).

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